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GENETIC ENGINEERING OF CLOSTRIDIUM DIFFICILE TOXIN A VACCINE

ANNUAL REPORT

Lycurgus L. Muldrow
Joe Johnson

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The long range goal of this project is to use C. difficile toxin A as a model to add technical information in the area of vaccine biotechnology using recombinant DNA techniques. This will be accomplished in part by predicting epitopes from the DNA sequence of toxin A, synthesizing DNA for these predicted regions, and testing their antigenic activity in expression vectors. Part of the toxin A gene has been cloned and sequenced. A total of twelve different epitopes have been predicted, synthesized, cloned into lambda gtll and tested for antigenicity. Alternative procedures for epitope mapping has been described and initiated.

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SUMMARY

Recombinant DNA technology allows for an approach to subunit vaccine production that should provide advantages over existing techniques. Improvement of vaccine biotechnology in the area of recombinant DNA studies using Clostridium difficile toxin A as the model, is the major long range objective of this project. This will be accomplished in part by predicting epitopes from the DNA sequence of toxin A, synthesizing DNA for these predicted regions, and testing their antigenic activity in expression vectors. Twelve regions representing the highest Antigenic Index peaks were selected and DNA oligomers for these corresponding regions were synthesized (fig. 1). Additional bases were added to these fragments in order to generate an EcoRI site. These synthetic single strain oligomers were then annealed and ligated into lambdagt11 and subsequently tested positive for the generation of clear plaques using X-gal. The clones were then tested with antiserum against toxin A to assay for antigenicity. Under the immunobolt conditions tested, peptides produced by these cloned fragments did not react with toxin A antibodies.

The 12 epitopes predicted represent the most probable antigenic regions using the IBI and MESQ software. These predicted an tested regions may not have been antigenic due to a variety of reasons. First, parameters for prediction did not allow for the selection of the appropriate epitopes. Second, the antigenic sites tested may represent discontinuous epitopes which cannot be selected using the above described procedures. Third, the antigenic site(s) from this particular system could not be expressed in the lacZ gene of λgt11. And finally, these AT rich DNA fragments were not stable in the λgt11 cloning system. Alternative procedures for epitope mapping has been described and initiated.

FOREWORD

Citations of commercial organizations and trade names in this report do not constitute an official Department of the Army endorsement or approval of the products or services of these organizations.

The investigator(s) have abided by the National Institutes of Health Guidelines for Research Involving Recombinant DNA Molecules (April 1982) and the Administration Practices Supplements.

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BACKGROUND

Recombinant DNA technology allows for an approach to subunit vaccine production that should provide advantages over existing techniques. The major long range objective of this project is directed toward the improvement of vaccine biotechnology in the area of recombinant DNA studies using Clostridium difficile toxin A as the model. This was proposed to be accomplished by using a unique series of recombinant DNA techniques to map epitopes.

1. C. difficile Toxin A Literature Review

C. difficile was first described in 1935 (1), but has only been recognized as a clinically significant pathogen within the last twelve years. This toxin producing nosocomial pathogen (2,3,4,5,6) is the causative agent for the diarrheal syndrome termed antibiotic associated pseudomembranous colitis (PMC) (7,8,9,10). PMC is a disease of the lower gastrointestinal tract that can be histopathologically characterized by exudative plaques on the bowel mucosa. If left untreated it can be fatal. The etiology of PMC does not only depend on colonization of C. difficile toxin producing strains, but other factors affecting the gastrointestinal tract may initiate the disease state, such as surgery, cancer chemotherapy and most frequently antibiotic therapy (11,12,13,14,15).

Pathogenicity and cytotoxicity associated with PMC has been linked to production of two toxins, A (enterotoxin) and B (cytotoxin). There have been a number of publications describing biological characteristics of toxins A and B (16,17,18,19,20,21,22) and evidence has developed to indicate that toxins A and B work synergistically to cause PMC (23,24).

Toxin A is a large protein that has been demonstrated to elicit a hemorrhagic fluid response in the rabbit intestinal loop assay, cause fluid accumulation in the suckling mouse assay (25,26), exhibit cytotoxic activity on mammalian tissue culture cells (27), and bind to and agglutinate rabbit erythrocytes (28). The molecular weight of toxin A has been reported by several different groups, using non-denaturing gels and gel filtration, to be approximately 440,000 to 600,000 (29,30,31). Studies directed toward the molecular genetics or DNA of C. difficile toxin A have been appearing in the literature in increasing numbers in the last several years. The first paper published on cloning part of C. difficile toxin A was reported by our group (32). Since this publication several other studies have followed on cloning, expression in E. coli, and sequencing of toxin A (33,34,35,36,37). We have also recently submitted for publication data that demonstrates that toxic strains of C. difficile normally contain the genetic composition for toxin A and B simultaneously (38).

The objective of this project is to use toxin A of C. difficile in a unique series of molecular procedures for the prediction, identification and purification of antigenic sites, which may ultimately be used as a model system for the development of subunit vaccines. Recombinant DNA technology allows for an approach to subunit vaccine production that should provide advantages over existing techniques. Potential advantages of genetically engineered antigens for the preparation of vaccines are stability, purity, safety of preparation, price, lack of side effects and variety of serotypes.

A number of determinants coding for antigens have been cloned from viruses, bacteria, parasites and toxins, with the long range goal of producing better vaccines and improving molecular techniques for the development of vaccines. Improvement of vaccine biotechnology in the area of recombinant DNA research is the major long range objective of this project.

2. First and Second Year Accomplishments

A genomic library in lambda gt11 of C. difficile chromosomal DNA was screened using anti-toxin A which resulted in the identification of one stable positive clone, lambda cd19. The insert in lambda cd19 was demonstrated to be a 0.3 kb fragment by restriction digestion, and by hybridization of the clone to a chromosomal digest of C. difficile. Verification of the immunological identity of the isolated toxin A gene fragment in lambda cd19 was determined by affinity purifying toxin A antibodies specific for lambda cd19 gene product, and using these selected antibodies to probe a Western blot of purified toxin A. After further biological characterization of this clone it was determined that this fragment contains an important antigenic region. The DNA of this fragment has been sequenced, and the amino acid sequence deduced.

This 0.3 kb toxin A positive fragment was demonstrated by Southern blot analysis to hybridize to a 16 kb fragment from a HindIII digestion of C. difficile chromosomal DNA. In an attempt to isolate the entire toxin A gene, the 16 kb HindIII fragment was cloned into λ2001. The HindIII fragment was cloned by digesting C. difficile DNA with HindIII, and separating the DNA fragments in the range of 12 to 20 kb using high pressure liquid chromatography. These restriction fragments were then ligated into the HindIII site of λ2001, and packaged in E. coli NM539. The recombinant phages were screened with a synthetic 40 bp probe which was derived for the sequence of the 0.3 kb fragment. A λ2001 recombinant which hybridized to the toxin A probe has been isolated and partially characterized.

MATERIALS AND METHODS

1. DNA Sequence Analysis

Sequence analysis of DNA encoding for toxin A was determined using the Sanger chain-termination sequence procedure. Protocols and reagents from the Sequenase™ kit (United States Biochemical Corporation, Cleveland, Ohio) were used for dideoxy sequencing. Taq DNA polymerase was used for high temperature chain termination DNA sequencing to read through compressions (16). The TAQuence™ System from United States Biochemical Corporation was used.

2. DNA Synthesis

Oligodeoxynucleotides were synthesized using beta-cyanoethyl phosphoramidite chemistry in a MilliGen 7500 DNA synthesizer (17). Analysis and purification of oligonucleotides were preformed by either separating DNA by polyacrylamide gel electrophoresis in 15% to 20% gels (18), or by fractionating DNA on a trityl-specific

reverse phase Delta Pak C18 (Waters Inc.) high pressure liquid chromatography (HPLC) column.

3. Screening Predicted Epitopes

Synthetic DNA oligomer were cloned and screened using the lambda gt11 expression system of Young and Davis (40) with the following alterations. The oligomers that were ligated into the EcoRI site of lambda gt11 were packaged with Packagene™ (Promega Biotec, Madison, WI), and the recombinants were directly screened without amplification. Toxin A antigen-producing plaques were monitored for on nitrocellulose filters using a 1:200 dilution of E. coli-lambda gt11 absorbed anti-toxin A, and the Bio-Rad horseradish peroxidase-bound goat anti-rabbit IgG immunoblot kit (Richmond, CA).

4. Restriction Digestion, Cloning and Screening

Restriction endonuclease digestion, and enzymatic manipulation of DNA for cloning were preformed as described by suppliers. Standard procedures that were used for isolation and manipulation of DNA for cloning are described in several molecular cloning manuals (19,20). Oligomers were radiolabeled by second strain synthesis using [alpha-^{32}P] dATP, and DNA hybridizations were performed as described earlier (15,19,20)

5. Computer Analysis

Nucleotide sequence data was analyzed by an IBM PC-XT computer with programs from International Biotechnologies, Inc., and a Digital Microvax computer using the data base and graphic programs of Cage/Gem (Battelle, Pacific Northwest Laboratories, Richland, Washington). Epitopes were predicted with the use of Pustell programs from International Biotechnologies, Inc., and the MSEQ programs from the University of Michigan.

6. PCR Amplification

Amplification of a 800 bp C. difficile toxin A gene fragment was carried out in a 50ul reaction volume containing 50ng of C. difficile DNA, 10mM Tris-HCl (pH 8.3), 50mM KCl, 1.5 mM $MgCl_2$, 2.5 units of Taq polymerase (Perkin Elmer, Norwalk, CT, USA), 200uM dNTP mix, and 0.2uM oligonucleotide primers. To ensure that all nuclease activity was destroyed, the reaction tube was heated for 5 minutes at 94°C prior to adding the Taq polymerase. Amplification was conducted for 37 cycles using a Perkin Elmer Cetus thermocyler (Norwalk, CT, USA). The cycle used to amplify the toxin A gene fragment consisted of denaturation at 94°C for 1 minute, annealing at 55°C for 1 minute, and extension at 65°C for 1 minute.

RESULTS AND DISCUSSION

During this past year a paper was published by C. H. Dove and coworkers (36)

on the entire C. difficile toxin A gene sequence. Based on the data published by this group, and data published by Von Ichil-Streiber's group (35) on overlapping toxin A DNA fragments which express antigenic sites, the DNA sequence of an 800 bp fragment has been identified which is thcugh to have a major antigenic site. This 800 bp fragment has also been identified to contain the 300 bp fragment that we earlier reported on cloning (32). Using the above information epitopes were predicted using the Antigenic Index of the IBI Pustell software program.

The antigenic index prediction method described by Jameson and Wolf (39) generates values for surface accessibility parameters and combines these values with those obtained for regional backbone flexibility and predicted secondary structure. The linear surface contour profile generated from these calculations allows for predicting potential continuous epitopes. Twelve regions representing the highest Antigenic Index peaks were selected and DNA oligomers for these corresponding regions were synthesized (fig.1). Additional bases were added to these fragments in order to generate an EcoRI site. These synthetic single strain oligomers were then annealed and ligated into λgt11 and subsequently tested positive for the generation of clear plaques using X-gal. The clones were then tested with antiserum against toxin A to assay for antigenicity. Under the immunoblot conditions tested peptides produced by these cloned fragments did not react with toxin A antibodies.

The 12 epitopes predicted represent the most probable antigenic regions using the IBI and MESQ software. These predicted an tested regions may not have been antigenic due to a variety of reasons. First, parameters for prediction did not allow for the selection of the appropriate epitopes. Second, the antigenic sites in this 800 bp fragment may represent discontinuous epitopes which cannot be selected using the above described procedures. Third, the antigenic site(s) from this particular system could not be expressed in the lacZ gene of λgt11. And finally, these AT rich DNA fragments were not stable in the λgt11 cloning system. This final explanation requires further discussion, because we have demonstrated that C. difficile toxin A DNA is highly unstable in lambda gtll.

From the original genornic library of C. difficile DNA, that was constructed in lambda gt11, approximately 35,000 plaques were screened for toxin A antigen-producing clones resulting in the original detection of 22 positive plaques. All but one of the identified positive plaques spontaneously lost the C. difficile inserts after plaque purification and amplification as indicated by immunologically testing for toxin A cloned antigens, and by reversion from clear to blue plaques. Lambda cd19 (the 300 bp TaqI clone) was the only recombinant plaque stable enough to allow for consistent immunological verification; however, as reported in the first annual progress report this clone lost one of the EcoRI cloning sites. It should be emphasized that this 300 bp TaqI fragment in lambda cd19 truly represents part of the toxin A gene. This has been verified by the numerous references that site the cloning of this fragment, and by the correlation of DNA hybridization patterns (33,34,35,36,41,42). However, the most convincing data that demonstrates the presence and location of this 300 bp cloned fragment comes from Dove's (36) DNA sequence of toxin A. The sequence data demonstrates that there is only one 300 bp TaqI fragment within the toxin A gene, which is located within a 4.5 PstI fragment, and a large HindIII fragment. Hybridization data published by our laboratory (32) demonstrated that [alpha-32P] labeled lambda

cd19 hybridized to a 4.5 kb fragment in a Pst1 chromosomal digestion of C. difficile, and a 16 kb fragment in a HindIII digestion (fig. 2).

Despite the apparent stability of lambda cd19, it mutated soon after the initial characterization of this clone. It was determined that this clone had significantly mutated after it lost its ability to produce a peptide that would react with toxin A antiserum, and after the DNA sequenced was compared to Dove's sequence (36). Consequently, it can be predicted that one possibility that a positive epitope could not be identified using the predicted cloned oligomers in lambda gt11 was due to the fact that C. difficile DNA is not stable in lambda gt11.

Through personal communication with numerous other investigators that use the lambda gt11 cloning system, it has become apparent that AT rich DNA is not stable in lambda gt11. C. difficile DNA is 70% AT rich.

In order to fulfill the contractual arrangement to assist in the development of the area of vaccine biotechnology using recombinant DNA techniques, and C. difficile toxin A as the model, an alternative expression system is currently being studied. In summary, fragments of C. difficile toxin A will be PCR amplified, subcloned into expression vectors, and tested for antigenicity using polyvalent antiserum made against toxin A. Upon identifying fragments that are immunoreactive, a series of overlapping exonuclease III generated deletion mutants will be produced and subcloned in the pATH3 expression vector (43,44). Selected N- and C-termini truncated fusion proteins generated from the deletion mutants will be tested by Western blotting to determine which mutants retained antigenic determinants. Upon narrowing down the region containing the epitope the exact sequence for the epitope will be identified by a series of different procedures including computer aided prediction, PCR site specific mutagenesis, and overlapping synthetic peptides. To date, the 800 bp fragment that has been identified to contain a major antigenic site has been PCR amplified with primers containing NotI sites, cloned (fig. 3), and expressed in the BlueScript expression system.

RECOMMENDATIONS AND CONCLUSIONS

One of the main objectives of this contract is to develop a system for testing epitopes using expression vectors such as lambda gt11, which includes reporting potential problems and solutions to these problems. From our experience of cloning toxin A, and as well as toxin B gene fragments in lambda gt11, and from personal communications with other investigators, we have identified a non-published common problems encountered when using this cloning system. AT rich DNA is not stable in lambda gt11. This cloning system as well as cosmids cloning systems should be avoided when the GC content of the DNA to be cloned is not similar to E. coli. By surveying the literature it appears that pBR322 derived vectors (including expression vectors) are more stable when using AT rich DNA.

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1.  5'  AA  TTC  CCT  AAT  GCT  CCT  TCA  AGA  G 3'
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               PRO  Asn  Ala  Pro  Ser  Arg
2.  5'  AA  TTC  AGA  TCA  TTG  GAA  AAT  GAC  G 3'
    3'       G  TCT  AGT  AAC  CTT  TTA  CTG  CTT  AA 5'
               Arg  Ser  Glu  Asn  Asp  Gly
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               Pro  Gly  Leu  Arg  Ser  Leu  Glu  Asn  Asp  Gly  Thr  Arg
        TAA  CTT  GAT  TCA  ATA  AGA  GAT  TAA  TAC  CCA  GGT  AAA  G 3'
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        Leu  Leu  Asp  Ser  Ile  Arg  Asp  Leu  Tyr  Pro  Gly  Lys.
5.  5'  AA  TTC  AGA  CCA  AGA  GAA  AAT  GAG   3'
               TCT  GGT  TCT  CTT  TTA  CTC  GTT  AA 5'
               Arg  Pro  Arg  Glu  Asn  Glu
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               AAT  AGA  G 3'
               TTA  TCT  CTT  AA 5'
               Asn  Arg
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          TTC  CTT  TTT  CAT  TTT  CTT  AA 5'
          Lys  Glu  Lys  Val  Lys
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          Arg  Thr  Ile  Asp  Gly  Lys  Lys  Tyr
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12. 5'  AA  TTC  GAT  GCT  AAC  AAT  ATA  GAA  G
    3'       G  CTA  CGA  TTG  TTA  TAT  CTT  CTT  AA 5'
               Asp  Ala  Asn  Asn  Ile  Glu
```

Figure 1. Epitopes Predicated for the 300 bp of Toxin A Gene.

Figure 2. Southern hybridization of restriction digested C. difficile chromosomal DNA with [alpha-^{32}P] dCTP labeled lambda cd19. C. difficile chromosomal DNA was digested with TaqI, lane 1; HindIII, lane 2; and PstI, lane 3.

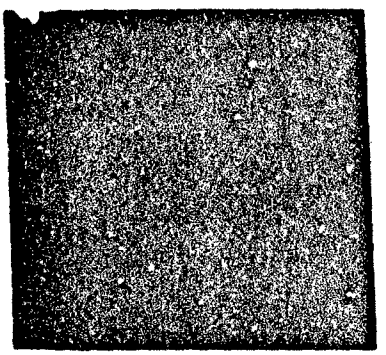

Figure 3. Toxin A DNA inserts digested from BlueScript hybrids. An 800 bp fragment was amplified from C. difficile strain 10463, and cloned into the NotI site of BlueScript. The five bands represent 300 bp fragments that were digested from five different hybrid BlueScript clones.

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